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PRINCIPAL INVESTIGATOR(S): Norman B. Javitt, M.D., Ph.D.

CONTRACTING ORGANIZATION: New York University Medical Center
New York, New York 10016

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13. ABSTRACT *(Maximum 200 words)*

Methods are in development for the analysis of bile acids in breast cyst fluid. For detection of bile acids at the femtomole level as a fluorescent ester linked through the 3-hydroxyl group common to most naturally-occurring bile acid, pyrene-1-carbonyl azide is under evaluation. Using known bile acid standards the fluorescent derivatives have been prepared under mild conditons and separated by reverse phase HPLC. However, decomposition of the azide and the instability of the isocyanate formed during the reaction gives a number of interfering fluorescent peaks. Fractionation of breast cyst fluid using a reverse phase C-18 cartridge provides an aliquot containing bile acids. However, unidentified fluorescent peaks also occur which may represent either artifacts or unidentified naturally-occurring compounds in breast cyst fluid. Resolution of the remaining analytical problems will permit analysis of 99 samples of breast fluid that have been obtained thus far.

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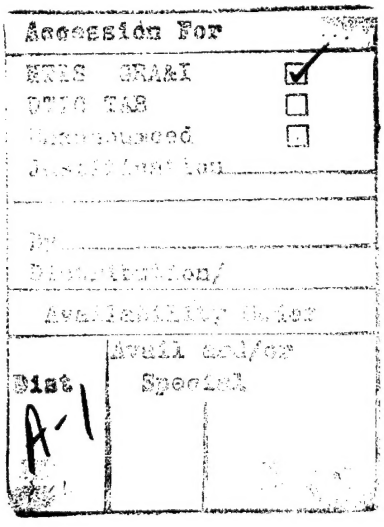

Lauren B. Jarrett 9/14/95
PI - Signature Date

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5. INTRODUCTION

Previous studies established the presence of high concentrations of bile acids in breast cyst fluid. The procedures that were used, particularly gas-liquid chromatography, required the preparation of volatile derivatives and by necessity, therefore, the use of chemical procedures that preclude the possibility of identifying the actual chemical state of the bile acid in the breast cyst fluid. Thus, it is not known if the lithocholic acid found in many samples of breast cyst fluid is actually present as either the taurine or glycine conjugate and also if it is esterified at the 3 position with glucuronic acid or as a sulfate. Thus, lithocholic acid could be present in breast cyst fluid in as many as 9 different chemical species (lithocholic acid, the 3-glucuronide or 3-ester sulfate, the glycine and taurine conjugates, either free or esterified with glucuronic acid or sulfate). Similarly other bile acids that are present could also have a minimum of 9 different species if one neglects the possibility that chenodeoxycholic acid may be esterified at either the 3 or 7 position.

To begin to tackle this analytical problem it was proposed that newer methods that would permit identification of the actual species that exists in breast cyst fluid be developed. This determination is important because it is known that each of the species of bile acids can have different biologic effects **[1]** and therefore a closer insight on the relationship to breast cancer can be obtained by identifying the actual species of bile acid present.

6. BODY: EXPERIMENTAL METHODS AND RESULTS

As indicated in the grant application our approach is to develop fluorescent derivatives of the bile acids that can be analyzed by HPLC.

REAGENT SELECTION: After review of the literature **[2,3]** we chose to evaluate pyrene-1-carbonyl azide (PCA) **[4]**. This reagent was chosen because:

a. the pyrene structure is likely to give the highest sensitivity for detection of the derivatives.

b. the formation of an adduct at the 3-hydroxy position common to all bile acids would detect all (1) unconjugated bile acids, (2) glycine-conjugated bile acids, and (3) taurine-conjugated bile acids. Bile acids esterified at the 3-position, such as glucuronides or sulfates, would not be detected. However, mild enzymatic treatment of the sample with β-glucuronidase and/or a sulfatase would selectively yield a free 3-hydroxyl group for formation of the PCA derivative. Thus virtually all the known species of bile acids that might be expected in breast cyst fluid could be detected.

PREPARATION OF THE REAGENT: Appendix page 1 indicates the method of synthesis of PCA from the commercially available acid. The

pyrene azide, kept in the refrigerator at -20°C underwent significant decomposition within a month.

PREPARATION OF DERIVATIVES: Appendix pages 2 and 3 indicate the reactions that occur in the formation of the PCA-3-ester derivative of sterols or bile acids. Reflux of PCA in benzene quickly converts the azide to the isocyanate. The isocyanate reacts with primary or secondary hydroxyl groups to form the pyrene ester. Although hydroxyl groups on the 7 and 12 position of the bile acid ring could theoretically form an ester, they are sterically much more hindered than at the 3 position.

ANALYSIS OF PYRENE ESTERS OF BILE ACIDS: Appendix pages 4, 5, and 6 indicate the yields of free and conjugated bile acids. It was found necessary to methylate the carboxyl group to obtain good reaction conditions. Also the presence of dichloromethane was found to greatly enhance ester formation. With 10-fold excess of PCA the reaction was virtually complete.

PREPARATION OF BREAST CYST FLUID FOR DERIVATIZATION: Appendix page 7 outlines the scheme for isolating the bile acids free of interfering substances by using a C-18 reverse phase column.

ANALYSIS OF BREAST CYST FLUID ENRICHED WITH BILE ACID STANDARDS: Appendix page 8 indicates the recovery of bile acids added to an aliquot of breast cyst fluid. Although recovery was satisfactory, the method of sample preparation gave too many peaks that overlap with the bile acid peaks.

The causes for these peaks are currently under investigation. In part they appear to be derived from (1) decomposition products of the PCA reagent, (2) impurities present in the C-18 packing, and (3) unknown naturally occurring compounds present in breast cyst fluid.

7. CONCLUSIONS

Although pyrene-1-carbonyl azide fulfills the criterion for sensitivity and provides a technique for forming a derivative via the free 3-hydroxyl group common to most of the naturally occurring bile acids, its use has a number of limitations. The reagent itself appears to be unstable, requiring frequent synthesis. The isocyanate is more unstable, needs to be freshly prepared, and yields a number of fluorescent decomposition products. For reasons that are not entirely clear esterification of the carboxyl group appears to be necessary for PCA-adduct formation to occur.

Other analytical problems relate to the isolation of the bile acid fraction from breast fluid. The use of a C-18 reverse-phase column has been a major advance in bile acid analysis. It is widely used to isolate bile acids from serum, urine, and feces. The presence, therefore, of so many unexplained peaks after derivative formation is surprising.

We are currently evaluating other fluorescent reagents and other methods of sample preparation.

We currently have 99 samples of breast cyst fluid, lyophilized and stored at -80°C awaiting bile acid analysis.

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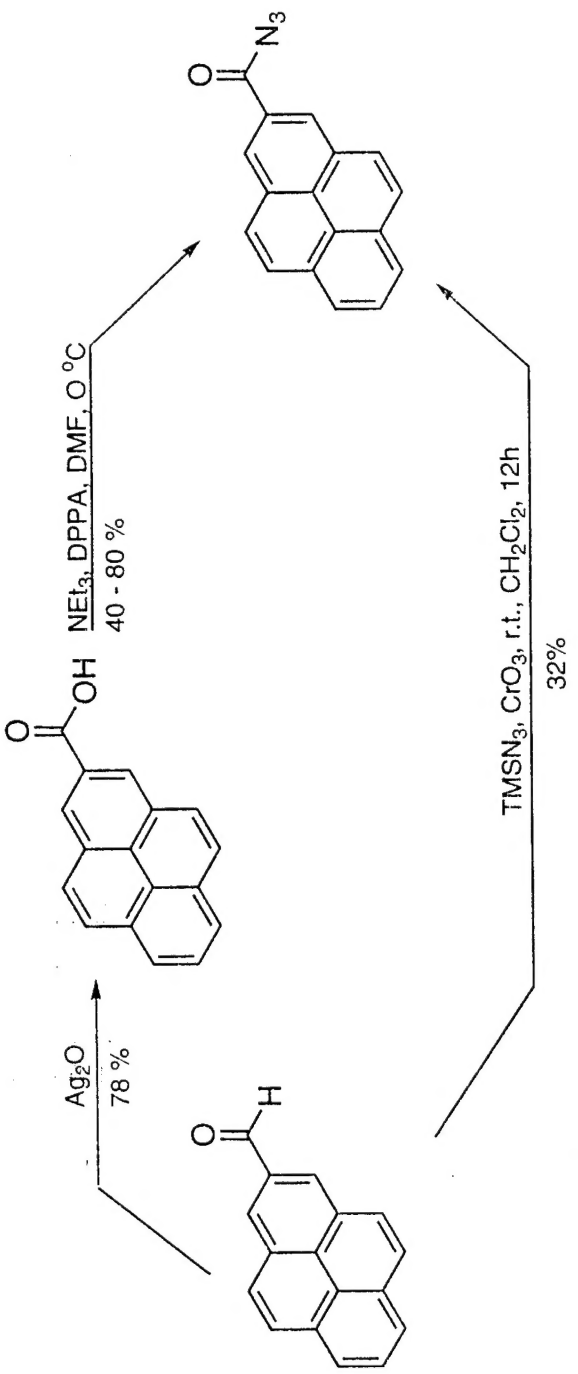
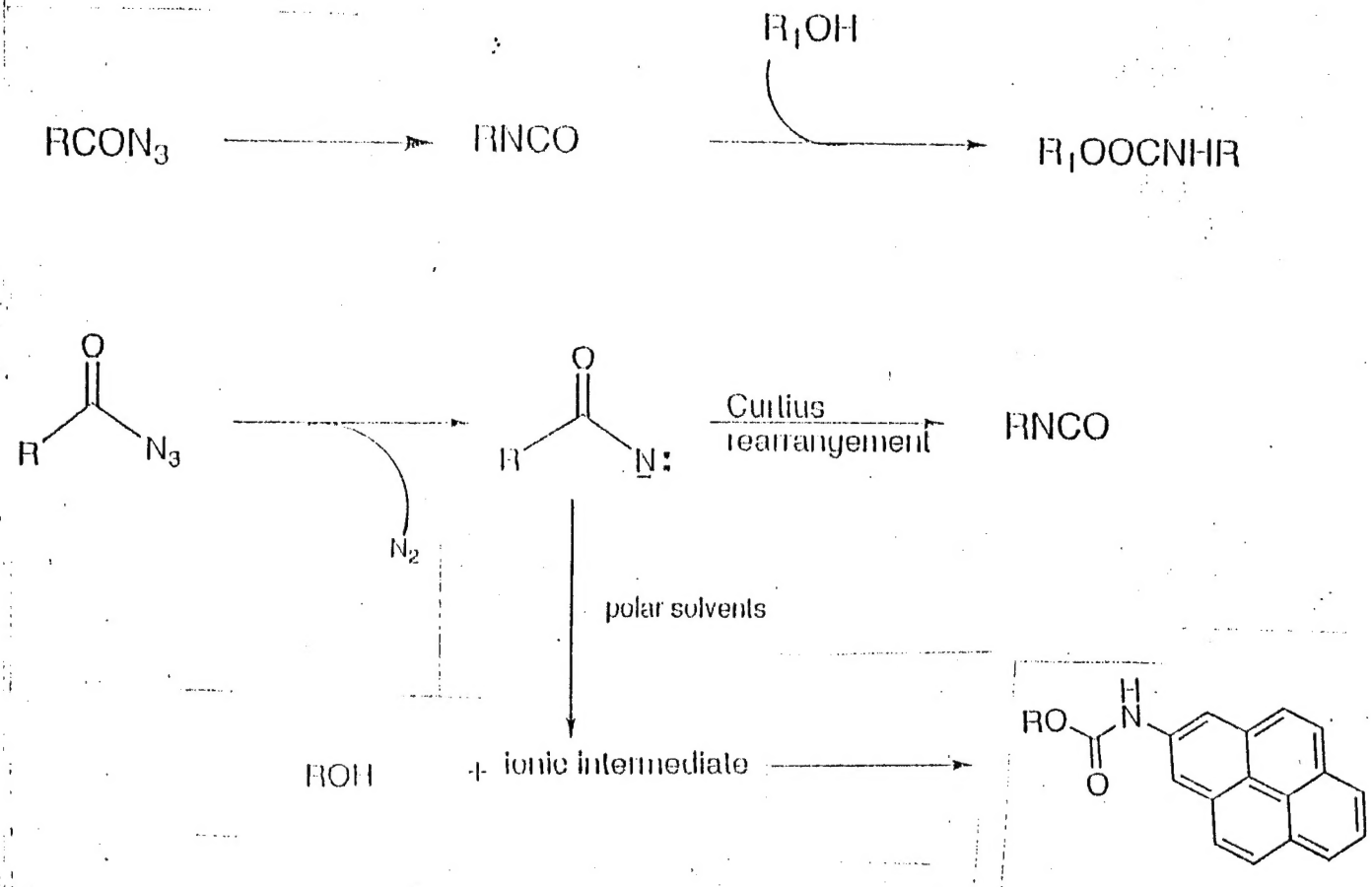
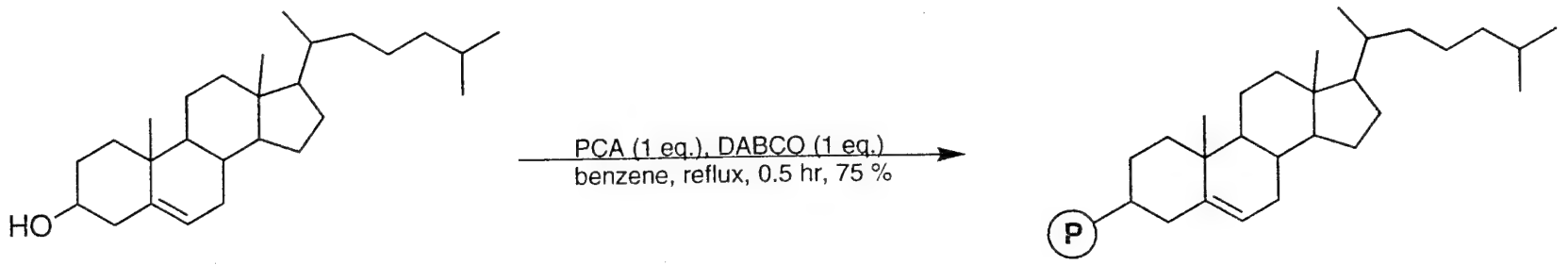
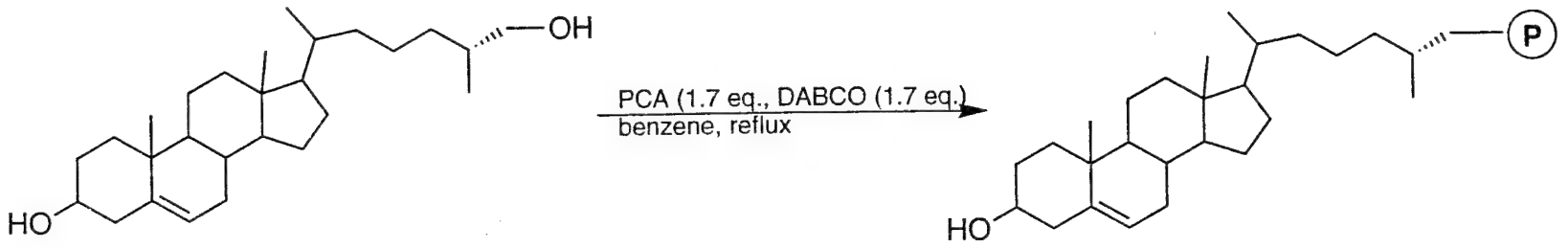
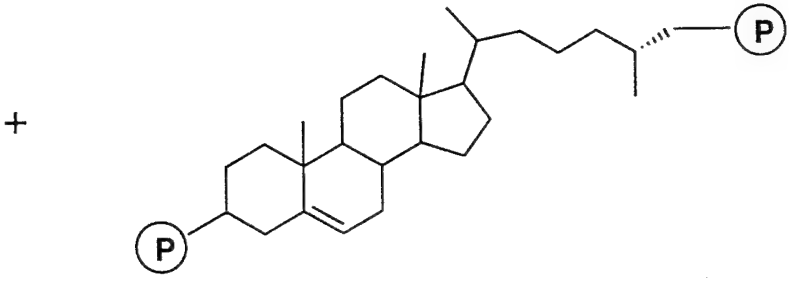


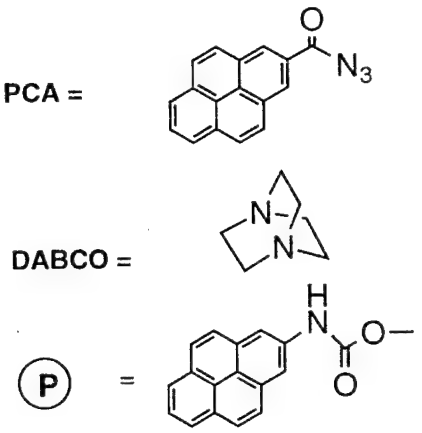
DPPA = $(PhO)_2P(O)N_3$

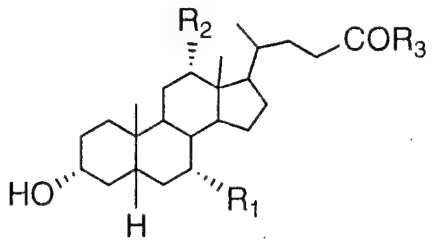

R1OH
RCON3
RNCO
R1OOCNHR
O
R
N3
N2
O
R
N:
Curtius
rearrangement
RNCO
polar solvents
ROH
+ ionic intermediate
RO
H
N
O


HO
PCA (1 eq.), DABCO (1 eq.)
benzene, reflux, 0.5 hr, 75 %
P

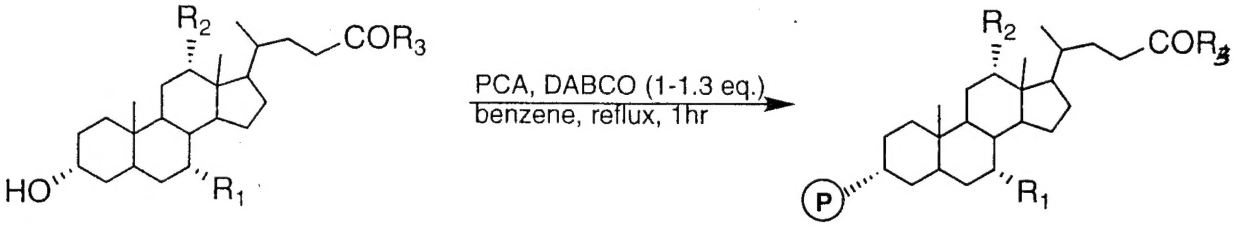

OH
HO
PCA (1.7 eq., DABCO (1.7 eq.)
benzene, reflux
P
HO


+
P
P

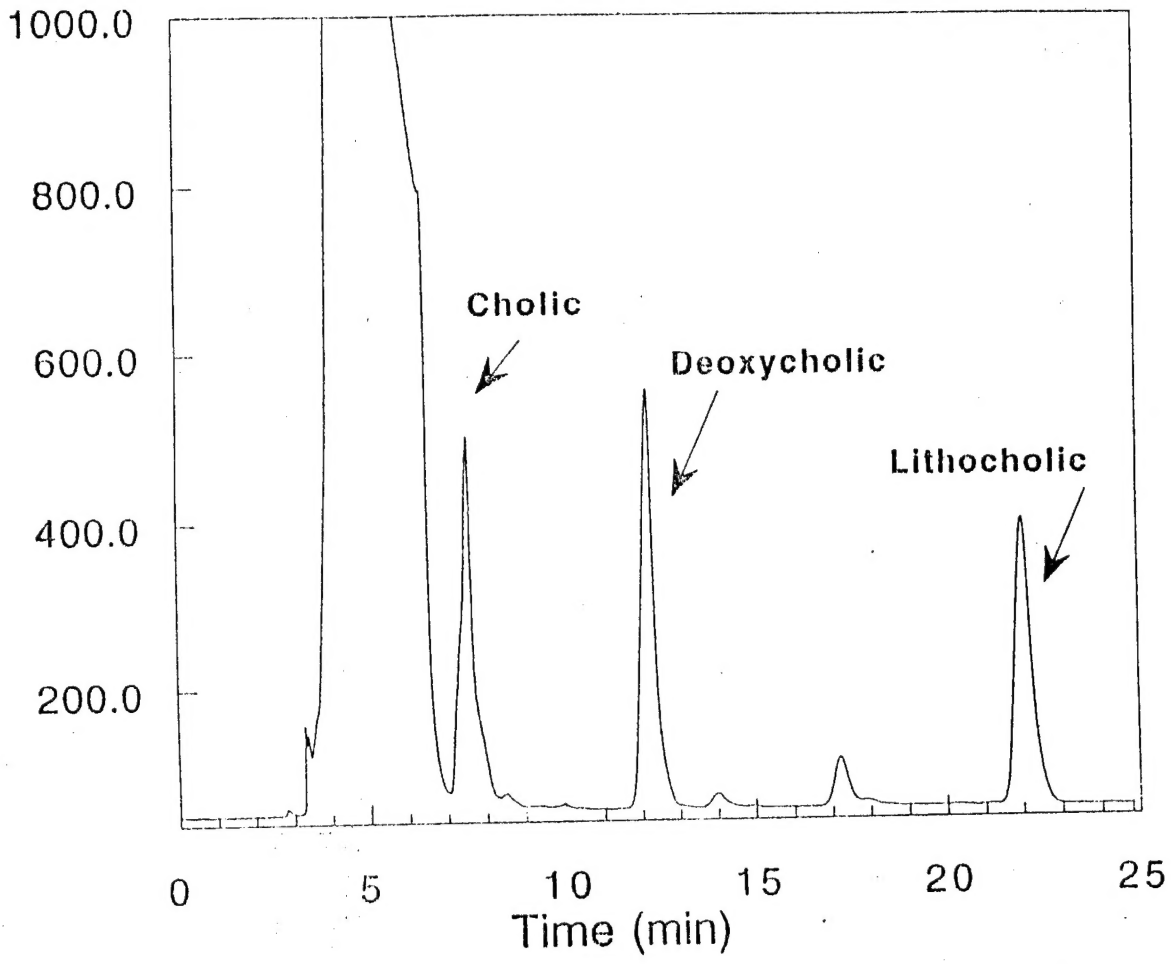

PCA =
O
N3
DABCO =
N
N
P
=
HN
O
O



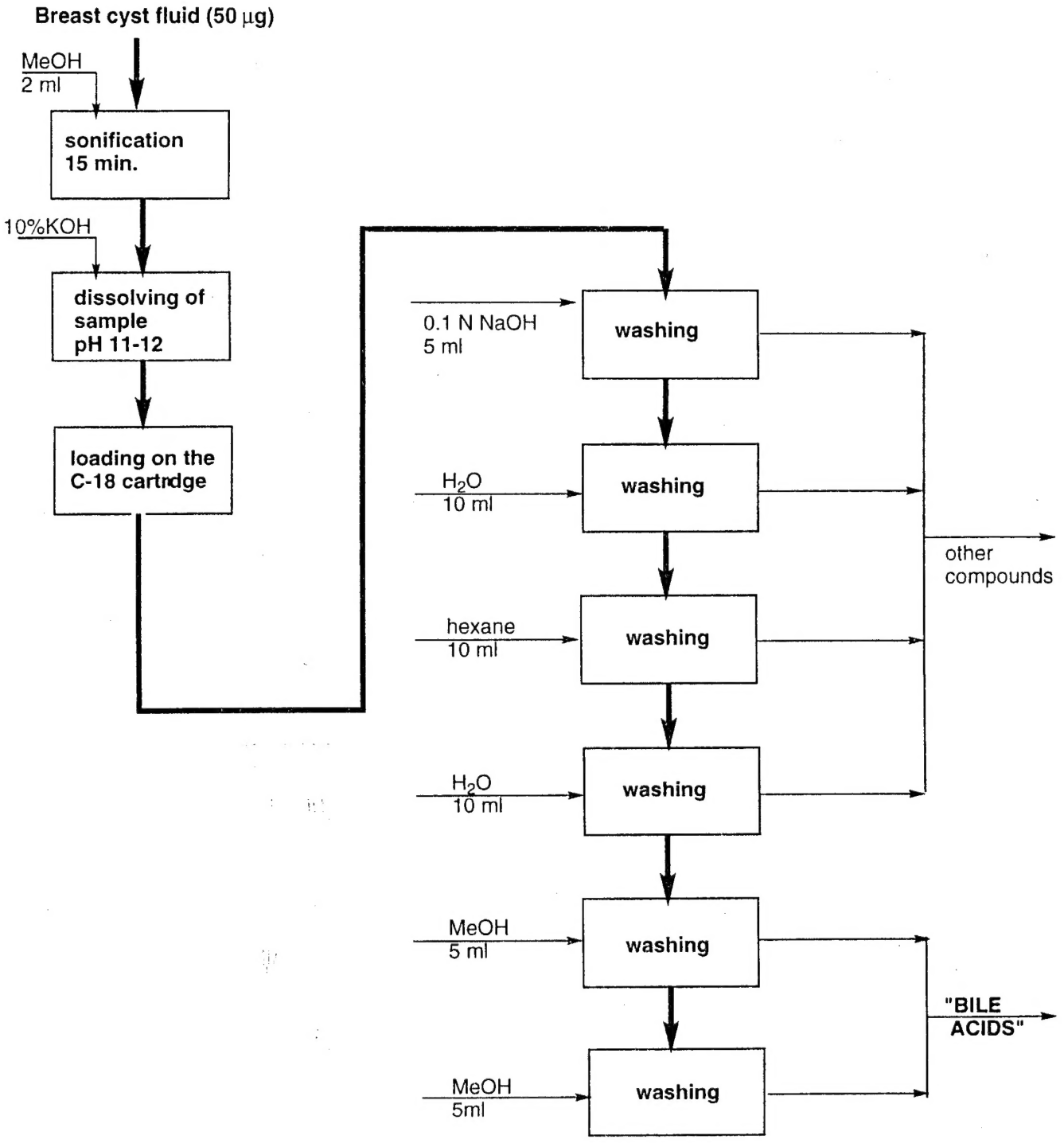
R_1	R_2	R_3	name of acid
OH	OH	OH	cholic
H	OH	OH	deoxycholic
OH	H	OH	chenodeoxycholic
H	H	OH	lithocholic
OH	OH	$NHCH_2CH_2COOH$	glycocholic
OH	OH	$NHCH_2CH_2SO_3H$	taurocholic



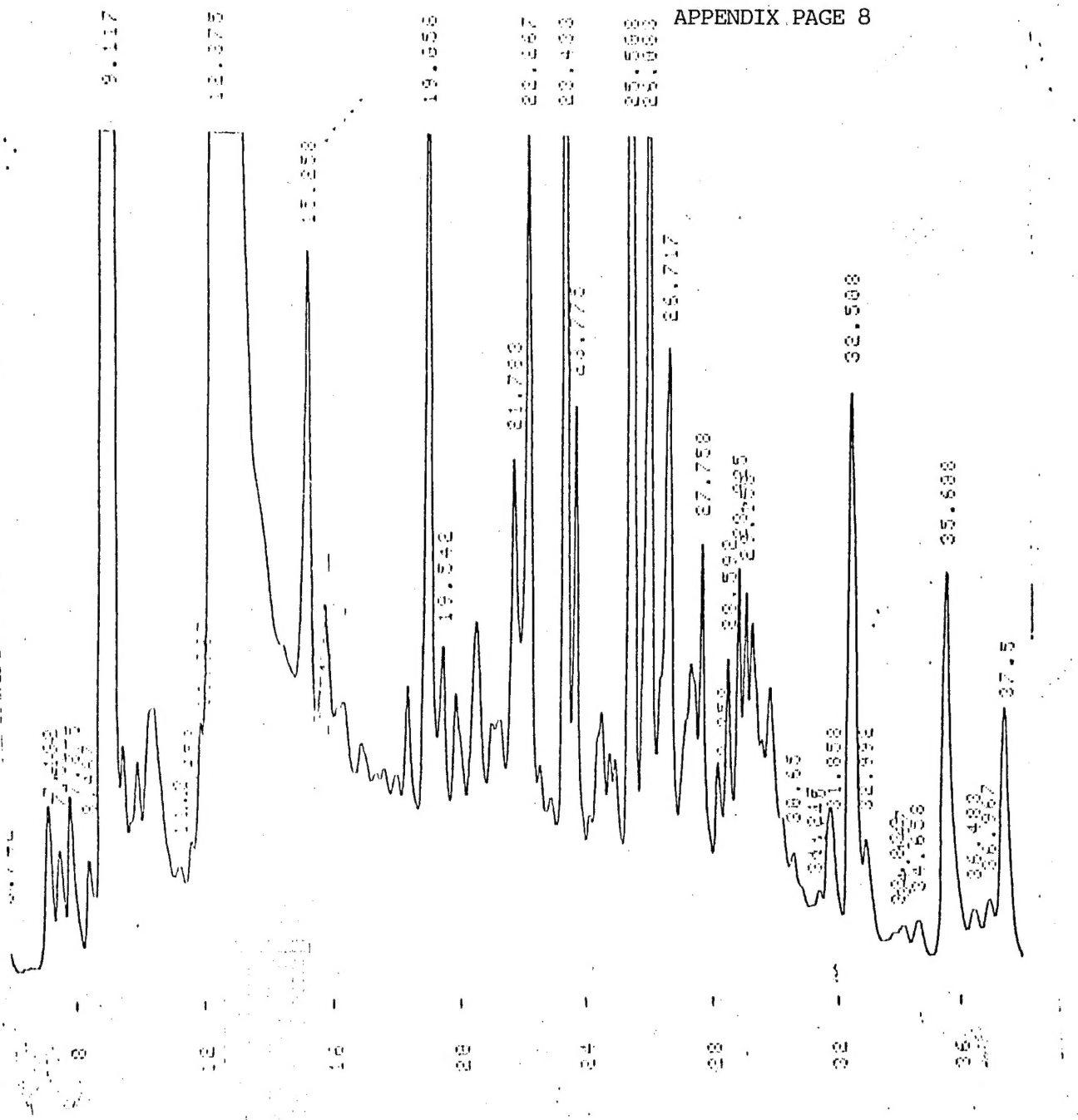
Entry	R_1	R_2	R_3	Yield of derivative
1	H	H	COOMe	22
2	H	OH	COOMe	81
3	OH	H	COOMe	not determined
4	OH	OH	COOMe	19
5	H	H	$NHCH_2COOMe$	
6	H	OH	$NHCH_2COOMe$	
7	OH	H	$NHCH_2COOMe$	42
8	OH	OH	$NHCH_2COOMe$	44
9	OH	OH	$NHCH_2CH_2SO_3Me$	not determined



Conditions: 2 nmol (2×10^{-9} mol) of each methyl ester of bile acid, PCA 100 nmol (1×10^{-7} mol) and DABCO 120 nmol (1.2×10^{-7} mol) in 200 µl of CH_2Cl_2 were heated in 60 °C until complete evaporation of solvent. Then 100 µl of CH_2Cl_2 was added and 5 µl of aliquot were injected.



Preparation breast cyst fluid sample for derivatization



RECOVERY OF BILE ACID STANDARDS ADDED TO BREAST CYST FLUID
HPLC OF PCA DERIVATIVES AFTER ELUTION FROM C-18 COLUMN